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(54) Oligonucleoside alkyl or arylphosphonate derivatives capable of crosslinking with or cleaving nucleic acids.

(57) A composition for inactivating a target nucleic acid which comprises an oligonucleoside alkyl or aryl-phosphonate analogue which is complementary to the sequence of the target nucleic acid and includes a functional group which reacts with the target nucleic acid to render the target nucleic acid inactive or nonfunctional.

FIG. 5
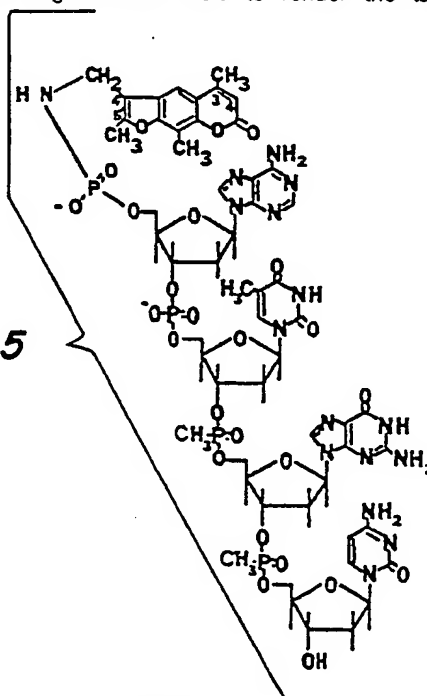
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OLIGONUCLEOSIDE ALKYL OR ARYLPHOSPHONATE DERIVATIVES CAPABLE OF CROSSLINKING WITH OR CLEAVING NUCLEIC ACIDS

The present invention is concerned with certain novel derivatives of oligonucleoside alkyl or arylphosphonates and uses thereof.

5   ## Background to the Invention

There has been previously disclosed the preparation of oligodeoxyribonucleoside alkyl or arylphosphonates. See, for example, Applicant's United States patents 4,469,863, 4,507,433 and 4,591,614. Applicant has also described the use of these oligonucleoside alkyl or arylphosphonates for controlling or

10   interfering with the effect or function of foreign nucleic acid. See, for example, Applicant's United States patent 4,511,713.

In the above mentioned United States Patent No. 4,511,713, there is described a process for selectively controlling or interfering with the effect or function of foreign nucleic acid by determining the base sequence for the nucleic acid and then binding the nucleic acid with an appropriately prepared nonionic

15   oligonucleoside alkyl or arylphosphonate analogue which has a base sequence complementary to the indicated sequence of the foreign nucleic acid. The analogue can be used to control protein synthesis caused by the foreign nucleic acid, or the replication and expression of the foreign nucleic acids. These analogues may also be used to control virus messenger RNA translation or pre-messenger RNA processing. Inhibition of the Herpes simplex virus (type 1 and type 2) is also disclosed.

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Brief Description Of The Invention

The present invention is concerned with the provision of derivatives of the previously disclosed

25   oligonucleoside alkyl or arylphosphonates, especially oligonucleoside methyl phosphonates, which include one or more functional groups that can react with a targeted nucleic acid to render the same nonfunctional or inactive. The reaction may involve crosslinking with the nucleic acid, cleaving the acid or the like. In any case, the nucleic acid is rendered ineffective and thus controlled by the combination of a reactive functional group attached to complementary oligomers. While the parent (underivatized) oligomers are themselves effective for controlling selected nucleic acids as previously described, the derivatives contemplated herein

30   have been found to be more effective than the parent oligomers as evidenced, for example, by the fact that the derivatives can be used in substantially smaller amounts to obtain equivalent inhibitory effects.

The derivatives of the invention may be broadly described as oligonucleoside alkyl or arylphosphonates which have a base sequence that is complementary to the base sequence of the targeted nucleic acid of

35   concern and which includes a functional group reactive with the targeted nucleic acid to render the same targeted nucleic acid inactive or nonfunctional. Preferably the oligomer is an oligonucleoside methyl phosphonate although other alkyl or arylphosphonates may be used.

A wide variety of functional groups are contemplated for use herein. According to one advantageous embodiment, the functional group is photoreactable, e.g. activated by ultraviolet light, for reaction with the

40   nucleic acid so as to effect the desired crosslinking between the oligomer and the nucleic acid. Alternatively, the functional group is one which cleaves the nucleic acid. In another alternative, the functional group may be an alkylating agent which on reaction separates from the oligomer and attaches to the targeted nucleic acid to render the same nonfunctional. In all of these embodiments, the oligomer, with its base sequence complementary to the nucleic acid of interest, binds itself to the targeted nucleic acid and

45   so positions its functional group as to enable the group to effectively react in one way or another with the nucleic acid to inactivate the same.

The positioning of the functional group on the oligomers may be varied. For example, it may be positioned at an end of the oligomer or intermediate the ends as may be most advantageous in the circumstances and taking into account the nature of the nucleic acid. A plurality of functional groups may

50   also be included on the oligomer although this is not usually necessary.

Representative of the embodiment of the invention where crosslinking occurs are derivatives of olicodeoxyribonucleoside methylphosphonates modified to include an aminomethyltrimethyl psoralen group (AMT). This group is photoactivatable but it will be appreciated that other crosslinking groups, which may or may not be photoreactable, may be used in lieu of the psoralen group.

A typical derivative for cleaving the nucleic acid is a transition metal chelating complex, e.g. ethylene diamine tetracetate (EDTA) or derivative thereof. However, cleaving groups other than EDTA are also contemplated e.g. phenanthroline, porphyrin or bleomycin.

The invention is applicable to any type of nucleic acid including double stranded DNA. However, it is of particular advantage for use with RNA, especially messenger RNA (mRNA) and pre-messenger RNA (pre-mRNA).

Description Of The Drawing

Various aspects of the invention are illustrated in the accompanying drawings wherein:

Figure 1 shows the structure of an oligonucleoside methylphosphonate d-ApTpGpC prior to its being derivatized according to the invention. The symbol p represents (3'-5') methylphosphonate internucleoside bond;

Figure 2 shows the partial nucleotide sequence of the initiation codon regions of Vesicular stomatitus virus N, NS and G protein mRNAs. The sequence of the complementary oligonucleoside methyl-phosphonate is shown below each mRNA sequence;

Figure 3 is a graph showing the effect of d-ACAGACAT on Vesicular stomatitis virus (VSV) protein synthesis in VSV-infected mouse L-cells;

Figure 4 shows the partial nucleotide sequence of the splice junctions of SV40 large T-antigen pre-mRNA, the sequence of the methylphosphonate oligomer complementary to the donor splice junction being shown below the pre-mRNA sequence;

Figure 5 shows the structure of the oligodeoxyribonucleoside methylphosphonate of Figure 1 derivatized with trimethylaminomethylpsoralen: d-AMT-pApTpGpC;

Figure 6 demonstrates photocrosslinking of (32p) labeled d-AMT-pTpGCACCAT with rabbit globin mRNA after irradiation;

Figure 7a illustrates a partial nucleotide sequence of α-globin mRNA showing a proposed secondary structure (Pavlakis et al, Cell, 19, 91-102, (1980) and the binding site for d-AMT-pTpGTTGGTC, the amino-methyltrimethylpsoralen group (AMT) being represented by the circle;

Figure 7b shows a partial sequence of β-globin mRNA with proposed secondary structure per Pavlakis et al and the binding site for d-AMT-pTpGCACCAT, the AMT group being represented by a circle;

Figure 8a is an autoradiograph showing the formation of photoadducts between rabbit globin mRNA and d-AMTpTpGTTGGTC or d-AMTpTpGCACCAT: shows the photoreactions of d-AMTpTGTTGGTC and d-AMTpTpGCACCAT;

Figure 8b is another autoradiograph which Figure 8c illustrates a psoralen-derivatized oligodeox-yribonucleoside methylphosphonate crosslinked with a uridine residue of mRNA;

Figure 9 shows the formation of photoadducts between rabbit globin mRNA and d-AMT(CH₂)-₂NHpTpGTTGGTC or d-AMT(CH₂)₆NHpTpGTTGGTC;

Figure 10 structurally illustrates an EDTA-derivatized oligodeoxyribonucleoside methylphosphonate;

Figure 11 shows an EDTA-Fe²⁺-derivatized oligonucleoside methylphosphonate (EDTA-Fe²⁺-d-A pTpGpC);

Figure 12 illustrates a synthesis scheme for the preparation of the EDTA-Fe²⁺ modified oligonucleoside methylphosphonates; and

Figure 13 is a photograph showing hydrolysis of rabbit globin mRNA by EDTA-Fe²⁺ modified d-TpGCACCAT.

With more specific reference to the drawings, Figure 1 illustrates a typical oligonucleotide analog which prior work has shown can be taken up intact by cells and which can be derivatized for purposes of the present invention. As shown in Figure 1, these analogs contain a 3'-5' linked methylphosphonate inter-nucleotide bond which replaces the phosphodiester bond found in naturally occurring nucleic acids. The methylphosphonate group is non-charged. As a result, the oligomer is quite lipophilic and is able to penetrate the plasma membrane of cells. The methylphosphonate linkage is also resistant to nuclease hydrolysis and therefore the oligomers have very long halflives in cell culture medium and inside cells. Previous studies with analogs ranging from three to seven nucleoside units in length have shown that they can inhibit tRNA aminoacylation and mRNA translation in cell-free systems, and that they can selectively inhibit protein synthesis in permeable E.coli cells. See Biochemistry 20, pages 1874-1880 (1980) and Proc. Natl. Acad. Sci. 78, pages 1537-1541 (1981). Solid phase synthetic procedures are known which permit the synthesis of oligomers of up to 15 nucleoside units in lengths. See, for instance, Nucleic Acids Research,

11, pages 5189-5204 and 6225-6242 (1983). Methods for characterizing the chain length and sequence of the oligomers and their interaction with mRNA are also available (Murakami et al, Biochemistry 24, (1985). These methods can be used to prepare sequence specific oligodeoxyribonucleoside methylphosphonates complementary to functional regions of virus mRNAs for present purposes.

Detailed Description Of The Invention

As will be appreciated, the invention contemplates modifying the previously known type of sequence specific oligomers to provide derivatives which will crosslink or otherwise react with nucleic acids, e.g. mRNAs to render them functionally inactive.

The invention is illustrated herein using psoralen or EDTA-derivatized oligodeoxyribonucleoside methyl-phosphonates. However, as indicated earlier, the invention contemplates the use of a wide variety of functional groups on the oligomers to react in any one of several ways (crosslinking, cleaving, alkylation) with the nucleic acid. Psoralen is typical of the photochemically reactive derivatives which are contemplated for use although other such groups, e.g. angelicin, reactive with the nucleic acid at appropriate wavelengths (e.g. 350-500 nm) may also be used. Substituents capable of alkylation reaction, especially methylation, which do not require photochemical activation for reaction with the nucleic acid may also be used. In this case the alkylating group may or may not leave the oligomer when it attaches to the nucleic acid to inactivate the same. Alkyl halides, haloacetamides such as bromoacetamide, phosphotriesters, etc. may be mentioned as alkylating agents for use herein.

It is also possible to include other functional groups, e.g. a radioactive isotope on the oligomer for reaction with the nucleic acid.

The EDTA-type derivatives function by cleaving the nucleic acid. As noted, EDTA is preferred for use but other nitrogen containing materials, e.g. azo compounds or nitreens, or transition metal chelating complexes referred to earlier, may be used.

When EDTA is used, iron is also advantageously tethered to the oligomer to help generate the cleaving radicals. The use of EDTA-iron to cleave nucleic acids has previously been disclosed. See Proc. Natl. Acad. Sci., USA, Vol. 82, pages 963-967 and 968-972, February, 1985. However, the concept of attaching the EDTA-iron to a complementary oligomer as contemplated herein to improve selectivity and to permit use in cells is novel and not obvious.

The length of the oligomer and the positioning of the functional group can be varied. The oligomer may comprise, for example, from 3-25 nucleosides and the functional group may be positioned along the chain where it will most effectively react with the nucleic acid. This will depend, in large measure, on the nucleic acid involved and its key site or sites although the optimum position can be readily determined for any particular situation by simple experiment.

It will be appreciated that the actual composition of the derivatives of the invention, e.g. the psoralen or EDTA derivatives, will vary depending on the base sequence of the oligonucleoside alkyl or aryl phosphonate, the sequence itself being determined by the nature of the nucleic acid. Typical derivatives which may be mentioned as exemplified herein include such psoralen-type derivatives as:

d-AMT-pApTGC
d-AMT-pTpGCACCAT
d-AMT-pTGTTGGTC
d-AMT-pTpGCACCAT
d-AMT(CH$_2$)$_2$NHpTpGTTGGTC
d-AMT(CH$_2$)$_4$NHpTpGTTGGTC
d-AMT(CH$_2$)$_6$NHpTpGTTGGTC
D-AMT-pTpGTTGGTCTTGT

Typical EDTA derivatives include, without limitation, d-EDTA-p-TpGTTGGTC and d-EDTA-pTpGCAC-CAT.

The derivatives of the invention may be readily prepared by adding the desired functional group, e.g., a photoactivatable or cleaving substituent, to an oligonucleoside methylphosphonate of the required sequence. The latter may be prepared as described in, for example, the above-mentioned U.S. patent 4,469,863 after which the crosslinking, cleaving or other functional group, e.g. psoralen, EDTA or an equivalent thereof, is attached to the appropriate position of the oligomer.

Before the starting material is prepared, it is, of course, essential to determine the sequence of the nucleic acid which is to be bound or cleaved with the methylphosphonate analogue or equivalent thereof. Such sequence can be determined in conventional fashion using methods and apparatus currently available to those in the art. The sequences for some viral, bacterial and mammmalian cellular nucleic acids are already well established but in other cases it will be necessary to determine these sequences, or at least an essential portion thereof, at the outset.

As noted earlier herein, it appears that the length of the nucleic acid sequence which is determined can be varied. Additionally, it appears that the positioning of the determined sequence in terms of the overall nucleic acid chain can also be varied although, generally speaking, it may be preferable to utilize a sequence at or near the 5' end of the chain or the middle thereof rather than the end as related to the function of the foreign nucleic acid in replication, expression, or translation processes. Normally the sequence determined, and the complementary analogue, should cover at least 3 adjacent bases and preferably 9-15 such bases. This may be varied, however, depending on other factors, e.g. whether a virus or bacteria or a malfunctioning cell is involved and the nature and effect of the nucleic acid of concern.

Once the sequence of the nucleic acid is determined the appropriate complementary alkyl or arylphosphonate analogue, preferably the methylphosphonate analogue, is prepared. This may be accomplished in known fashion by, for example, condensing or esterifying a selected nucleoside which has a 3'-OH group and a protected 5'-OH group with an alkyl or aryl phosphonic acid, the resulting product then being condensed with another nucleoside which has a protected 3'-OH group and a reactive 5'-OH group. The esterification reactions are continued with sequential reaction involving the phosphonic acid group of the preceding product and a selected nucleoside until the desired oligonucleoside is obtained after which protecting group or groups are removed to give the desired oligonucleoside alkyl or arylphosphonate analogue wherein the nucleoside units are linked by isoteric 3'-5' alkyl or arylphosphonate groups. These latter groups are preferably methylphosphonate linking groups although if desired the methyl substituent may be replaced by other alkyl or aryl groups or substituents.

As noted, it is essential for the phosphonate analogue to have a sequence complementary to the foreign nucleic acid involved or targeted. Thus, for example, in the case of a virus DNA where the sequence may be -A-T-C-G, the analogue must have the complementary sequence of -T-A-G-C-so that the sequences are bound together according to the Watson-Crick theory as follows:

$$
\begin{array}{c}
-A-T-C-G- \\
|\quad|\quad|\quad| \\
-T-A-G-C-
\end{array}
$$
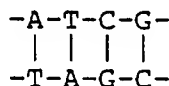
Likewise in the case where mRNA having the sequence -UAGC-is concerned, the complementary analogue would be -ATCG-.

It will be appreciated that the oligomer serves two functions, i.e. it exhibits an inhibiting effect of its own on the nucleic acid and it helps to selectively position the functional group at the appropriate spot for most effective reaction with the nucleic acid. The oligomer itself may remain attached to the reactive substituent or the oligomer may split off when the functional substituent reacts with the nucleic acid. In both cases, the oligomer functions to appropriately position the functional or reactive component.

Psoralen is photoreactable, as noted, and requires ultraviolet light for reaction with the nucleic acid. When a photoactivable group is used, it is important to use one which will crosslink or react with the nucleic acid on exposure to light (including laser) but not photodegrade. It appears that any α' β-unsaturated ketone substitution which is ultraviolet light reactable can be used for present purposes. An advantage in using psoralen however, is that it.appears to be free from side reactions and any alternative thereto should also preferably be specifically reactive with the nucleic acid.

While various modifications are contemplated, the preferred derivatives of oligonucleoside methylphosphonates contemplated herein contain psoralen or EDTA linked to the 5'-end of a deoxyribooligonucleoside methylphosphonate. The psoralen derivatives have been shown to covalently crosslink to the target mRNA on activation with ultraviolet light to inhibit translation of the mRNA. This inhibition has been obtained at very low concentrations (2.5 μM) of oligomer and represents a marked improvement (about 80 fold) in efficiency over that of non-derivatized oligomers of the same nucleotide sequence.

The EDTA-derivatives selectively cleave their target nucleic acid to render it biologically nonfunctional. Such derivatives have been shown to cleave mRNA at extremely low oligomer concentrations (~ 0.1 μM). This indicates that the EDTA derivative has potential as an antiviral or chemotherapeutic agent.

5

Both types of derivatized methylphosphonate oligomers, i.e. the crosslinking type represented by the psoralen derivative and the cleaving type represented by the EDTA derivative are particularly useful to molecular biologists and virologists in enabling them to study gene expression in a selective and well defined manner. Thus in addition to their potential value as chemotherapeutic agents, the present derivatives are useful in basic research, and particularly with respect to the control of gene expression.

The invention and advantages thereof are illustrated by the following examples wherein Examples 1 and 2 are given only for comparison purposes while Examples 3-6 are illustrative of the invention.

Example 1

Inhibition of VSV mRNA Translation by Oligodeoxyribonucleoside Methylphosphonates (Underivatized)

Methylphosphonate oligomers complementary to the initiation codon regions of Vesicular stomatitis virus (VSV) N, NS and G protein mRNAs were synthesized (see Figure 2). The results of studies with d-ApACAGACAT which is complementary to the N protein mRNA are shown in Table 1. At low concentrations (50 to 100 μ M), the oligomer selectively inhibits N protein synthesis when N, NS and M protein mRNAs are translated simultaneously in a reticulocyte lysate.

The effects of these oligomers on the synthesis of the five VSV proteins in VSV-infected mouse L-cells were determined. Figure 3 shows the results obtained with d-ApACAGACAT which is complementary to N protein mRNA. In contrast to the results obtained in vitro, the oligomer inhibits synthesis of all five virus proteins to approximately the same extent. At these concentrations, the oligomer has no detectable inhibitory effects on cellular protein synthesis by mouse L-cells and is not cytotoxic to the mouse L-cells as determined from mass culture growth curves. Thus the methylphosphonate oligomer appears to specifically inhibit virus function. The observation that synthesis of all five virus proteins is inhibited may be due to the known requirement of N protein for synthesis of VSV proteins in infected cells.

Table 1

Effect of dApACAGACAT on Translation of Vesicular Stomatitis Virus mRNA at 30°C in a Rabbit Reticulocyte Lysate

Oligomer Concentration	% Inhibition(a)		
μM	N	NS	M
50	23	−4	−16
100	33	−1	−19
150	77	38	43

(a) Negative sign indicates stimulation of synthesis.

Similar selective inhibition of VSV versus L-configuration protein synthesis was also obtained with the methylphosphonate oligomers complementary to the NS and G protein mRNAs. Again inhibition of all five virus proteins was observed. In addition to their effects on VSV protein synthesis, each of the oligomers reduce virus titer approximately one log unit at an oligomer concentration of 150 μM.

The results of these experiments indicate that mRNA translation can be selectively controlled in virus infected cell using the methylphosphonate oligomers.

Example 2

Inhibitory Effects of Oligodeoxyribonucleoside Methylphosphonates Complementary to the Splice Junction of Virus Pre-mRNA

To determine the possibility of controlling gene expression at the level of mRNA processing, oligonucleoside methylphosphoates have been synthesized which are designed to interact with the donor and acceptor splice junction sequences of SV40 large T-antigen precursor mRNA (pre-mRNA). The methylphosphonate oligomers shown in Figure 4 which are complementary to the donor splice junction of SV40 large T-antigen and to the 5'-terminal sequences of U₁ RNA were tested. U₁ RNA has been implicated as an agent involved in the splicing of mammalian and viral pre-mRNAs (Nature 283, pp. 220-223, 1980). The effects of the compounds on large T-antigen synthesis in SV40-infected African green monkey kidney cells (BSC40) were determined. The level of large T-antigen was measured by an immunoprecipitation technique using a monoclonal antibody directed against large T-antigen. The splice junction-complementary oligomer, d-AATACCTCA, and the two U₁ RNA-complementary oligomers each reduce the level of large T-antigen in the cells, while the non-specific oligomer, d-TTTTTT, had no effect. None of the oligomers had any effect on overall protein synthesis by the BSC40 cells.

A methylphosphonate oligomer, d-TpCCTCCTG, which is complementary to the acceptor splice junctions of Herpes simplex virus type 1 immediate early mRNA 4 and 5 was also prepared. The immediate early proteins are believed to be necessary for regulating the early and late genes of HSV-1 during infection. The oligomer was tested for its effects on HSV-1 in virus infected Vero and human cells. Virus growth was inhibited two log units by 300 μM oligomer. The oligomer selectively inhibits HVS-1 protein synthesis and DNA synthesis in the infected cells. It has no appreciable effect on cellular protein or DNA synthesis. Of particular interest is the effect of d-TpCCTCCTG on the virus proteins. Immediate early mRNA 4 codes for a 68 K protein. This protein is virtually absent in virus infected cells treated with the oligomer.

Example 3

Oligodeoxyribonucleoside Methylphosphonates Which Crosslink with mRNA

In the experiments described in Examples 1 and 2, rather high concentrations, 100 to 300 μM, of oligomers were required to obtain significant inhibitory effects. This is due to the equilibrium nature of the interaction between the oligomer and its target mRNA. According to one aspect of the present invention, more effective inhibition is obtained if the oligomer is crosslinked with the mRNA. This is shown in the present example.

Oligonucleoside methylphosphonates derivatized at their 5'-end with aminomethyltrimethylpsoralen (AMT), a photoactivatable crosslinking reagent, were prepared. The structure of one such derivatized oligomer is shown in Figure 5.

The derivatized oligomer d-AMT-pTp-GCACCAT which is complementary to the initiation codon region of rabbit β globin mRNA and partially complementary to the initiation codon region of α globin mRNA, was prepared. The AMT group is opposite a U residue when the oligomer is complexed with β globin mRNA, whereas it lies opposite a G residue when complexed with α globin mRNA. Since the 3,4 double bond of psoralen can form cyclobutane type adducts with the 5,6 double bonds of U and C residues upon irradiation with 365 nm light, the d-AMT-pTpGCACCAT is able to form a photoadduct with β globin mRNA.

When a mixture of [^{32}p]-labeled d-AMT-p-TpGCACCAT and rabbit globin mRNA were photoirradiated and then subjected to agarose gel electrophoresis, radioactivity was observed to migrate with the mRNA (see Figure 6). No radioactivity was observed in the position of mRNA in the absence of irradiation or in the absence of mRNA. No degradation of the mRNA as a result of irradiation was detected in these experiments. These results are consistent with covalent bond formation between the mRNA and the d-AMT-pTpGCACCAT .

With reference to Figure 6, it is noted that this illustrates photocrosslinking of [^{32}p]-labeled d-AMT-pTpGCACCAT (0.0567 μM) with rabbit globin mRNA (0.150 μM) after irradiation at 0°C. The reaction mixtures were electrophoresed on a 2.5.% agarose gel. Lanes 1-3: Oligomer and mRNA irradiated for 40, 80 and 120 min. respectively. Lanes 4-7: Oligomer and mRNA were preannealed for 30 sec. at 100° and then irradiated for 0, 40, 80 and 120 min. respectively. The positions of the top of the gel (←) and the mRNA are indicated.

Example 4

The comparative effect of d-AMT-pTpGCACCAT and d-TpGCACCAT on mRNA translation was also determined. After photoirradiation, translation was inhibited 75% (β globin) and 56% (α globin) by only 25 μM AMT-pTpGCACCAT. In contrast, 25 μM d-TpGCACCAT had no inhibitory effect. Comparable inhibition by d-TpGCACCAT, 70% β globin, 73% α globin, was observed at 200 μM concentration.

The results of the experiments given in Examples 3 and 4 indicate that crosslinking is an effective way to increase the efficiency and selectivity of inhibition by methylphosphonate oligomers. In addition, covalent bond formation between the oligomer and target mRNA offers a way for studying the mechanism of inhibition in a definitive manner.

The results of the foregoing also indicate that the derivatized oligodeoxyribonucleoside methyl-phosphonates can be used to specifically control gene expression in living cells by interfering with translation or splicing of mRNA. Because the specificity of these compounds resides in their ability to bind to complementary nucleic acid sequences, it should be possible to use nucleic acid sequence information to design novel derivatives of methylphosphonate oligomers which can be used to probe the function of specific proteins in normal, transformed or virus-infected cells. It may also be possible to use these analogs as anti-viral or chemotherapeutic agents.

Example 5

The structures and binding sites for AMT-derivatized oligomers which are complementary to either α and β globin mRNA are given in Table II and illustrated in Figures 7a and 7b. As shown, the oligomers are each eight nucleotides in length and begin with the sequence pTpG. The AMT group is linked directly to the 5′ phosphoryl group via a phosphoramidate bond or via an aminoethyl or aminohexyl linker arm.

TABLE II

Structures of Aminomethyltri-methylpsoralen Oligonucleoside Methylphosphonate Derivatives

Oligomer*	Binding Site
d-AMTpTpGTTGGTC	α globin mRNA nucleotides (60-67)
d-AMTpTpGCACCAT	β globin mRNA nucleotides (54-61)
d-AMT(CH₂)₂NHpTpGTTGGTC	α globin mRNA nucleotides (60-67)
d-AMT(CH₂)₆NHpTpGTTGGTC	α globin mRNA nucleotides (60-67)

*AMT = aminomethyltrimethylpsoralen

The oligomers are complementary to either the coding region of α globin mRNA (nucleotides 60-67) or the initiation codon region of β globin mRNA (nucleotides 54-61). According to the secondary structure model of Pavlakis et al (Cell, 1a, 90-102, 1980) and as shown in Figures 7a and 7b, the physical binding sites for both oligomers lie in single stranded loop regions of the mRNAs. In each case the AMT group is lined up opposite a uracil residue in the mRNA which is the covalent binding site for the oligomer. In the case of the β globin binding site this uracil is hydrogen bonded to an A residue at the end of a base-paired stem region, whereas in α globin mRNA the uracil residue is free.

Irradiation of [³²p]-labeled AMT-oligomer and rabbit globin mRNA with 365 nm light at 4°C results in the formation of covalent adducts between the oligomer and mRNA. As shown in Figure 8a, d-AMTpTpGTTGGTC binds specifically to α globin mRNA while d-AMTpTpGCACCAT binds specifically to β globin mRNA. The identities of the two mRNAs in the gel were confirmed independently by hybridization with [³²p]-labeled oligonucleotides whose sequences were complementary to the initiation codon region of α or β globin mRNA. The material at and near the top of the gel represents aggregated α and β globin mRNA which did not move into the gel.

The oligomers themselves undergo a photochemical reaction when irradiated at 365 nm as shwon in Figure 8b. A new, faster moving material appears as the time of irradiation increases d-AMTpTpGCACCAT is almost completely converted after 30 min. whereas approximately 50% of d-AMTpTpGTTGGTC is reacted after 45 min. Examination of molecular models shows that the psoralen ring system can stack on the thymine base with the 4',5' double bond of the psoralen furan ring below the 5,6 double bond of the thymine. Proton NMR spectra of a model compound, d-AMTpT, suggest that such stacking does occur in aqueous solution. Irradiation of the molecule in this conformation could thus lead to cyclobutaine ring formation between the psoralen group and the thymine residue at the 5' end of the oligomer. When d-AMTpT is irradiated, the intensity of the UV spectrum diminishes. The UV spectrum is restored by irradiation at 254 nm. This behavior is consistent with the known properties of cyclobutane adducts formed between psoralen and thymine base residues (Cimino et al, Ann. Rev. Biochem. 54, 1151-1193, 1985).

As shown in Figure 8a, d-AMTpTpGTTGGTC appears to crosslink more efficiently with its target mRNA than does d-AMTpTp GCACCAT. This behavior may be the result of differences in the binding sites for the two oligomers. As shown in Figures 7a and 7b, the binding site for d-AMTpTpGCACCAT lies adjacent to a stem region. In order to crosslink to uracil #62, the stem must open up which may be energetically unfavorable. In contrast uracil residue #68 of α globin mRNA is free to crosslink with the psoralen of d-AMTpTpGTTGGTC.

The decreased efficiency of crosslinking by d-AMTpTpGCACCAT may also reflect its conversion to a new product, possibly intramolecular cyclobutane adduct, during the course of irradiation. A separate experiment showed that d-AMTpTpGCACCAT, which had been pre-irradiated at 365 nm for 45 min., underwent little or no crosslinking when it was subsequently irradiated in the presence of globin mRNA.

5 Thus the photoconversion of d-AMTpTpGCACCAT to an inactive product competes effectively with the crosslinking reaction resulting in a lowered efficiency of covalent adduct formation with the globin mRNA. Examination of molecular models shows that if an intramolecular cyclobutane type adduct is formed by d-AMTpTpGCACCAT, the psoralen ring of this adduct is now fixed in an orientation unfavorable to crosslinking with the uracil residue fo the mRNA strand.

10 The effect of linker length on the efficiency of the crosslinking reaction is not completely defined. Examination of molecular models suggest that a linker containing between 3 to 7 atoms facilitates crosslinking by allowing the 3,4 double bond of the psoralen pyrone ring to lie closer to the uracil 5,6 double bond. This increased proximity decreases the re-orientation of the uracil required for crosslinking.

The following additional details are provided with respect to Figures 8a and 8b:

15 Figure 8a shows the formation of photoadducts between rabbit globin mRNA and d-AMTpTpGTTGGTC (lanes 1-4) or d-AMTpTpGCACCAT (lanes 5-8). Aqueous solutions of mRNA (80 n$\underline{M}$) and [^{32}p]-labeled oligomer (100 n$\underline{M}$) were irradiated at 365 nm at 4°C for 5 min. (lanes 1,5) 15 min. (lanes 2,6), 30 min. (lanes 3,7), and 45 min. (lanes 4,8). The reaction mixtures were electrophoresed on a 7% acrylamide/0.25% agarose gel which contained 7 $\underline{M}$ urea at 500 V for 4 hrs. The gel was autoradiographed at -80°C for 16

20 hrs. The top of the gel and the positions of α and β globin mRNA, which were determined by staining the gel with ethidium bromide, are indicated at the side of the autoradiogram. The material in the lower part of the gel is unreacted oligomer.

Figure 8b shows the photoreactions of d-AMT-pTGTTGGTC (lanes 1-4) and d-AMTpTpGCACCAT (lanes 5-8). Aqueous solutions of the [^{32}p]-labeled oligomers were irradiated at 365 nm at 4°C for 5 min.

25 (lanes 1,5), 15 min. (lanes 2,6), 30 min. (lanes 3,7) and 45 min. (lanes 4,8). The reaction mixtures were electrophoresed on a 20% acrylamide gel which contained 7 $\underline{M}$ urea at 800 V for 2 hrs. The gel was autoradiographed at -80°C for 16 hrs. The position of the starting oligomers are indicated at the sides of the autoradiogram.

Figure 8c, as noted earlier, illustrates how a psoralen-derivatized oligodeoxyribonucleoside methyl-

30 phosphonate crosslinks with a uridine residue of mRNA.

As shown in Figure 9, d-AMT(CH₂)₂-NHpTpGTTGGTC and d-AMT(CH₂)₆NHpTp GTTGGTC both specifically crosslink with α globin mRNA at an oligomer concentration of 2.7 n$\underline{M}$. Separate experiments did not detect crosslinking with 2 n$\underline{M}$ d-AMTpTpGTTGGTC. Thus, the oligomers containing aminoalkyl linkers appear to crosslink more efficiently than the oligomer in which AMT is attached directly to the oligomer.

35 Because both oligomers shown in Figure 9 have the same specific activity, the results show that d-AMT-(CH₂)₂-NHpTpGTTGGTC crosslinks more efficiently than d-AMT(CH₂)₆NHpTpGTTGGTC. It appears that linker length and oligomer chain length have some effect on the efficiency of the photocrosslinking reaction.

A more detailed explanation regarding Figure 9 is as follows:

This figure shows the formation of photoadducts between rabbit globin mRNA and d-AMT(CH₂)-

40 ₂NHpTpGTTGGTC (lanes 1-5) or d-AMT(CH₂)₆NHpTpGTTGGTC (lanes 6-9). Aqueous solutions of mRNA (100 n$\underline{M}$) and [^{32}p]-labeled oligomer (2.7 n$\underline{M}$) were irradiated at 365 nm at 4°C for 0 min. (lane 1), 5 min. (lanes 2,6), 15 min. (lanes 3,7), and 30 min. (lanes 4,8). The oligomers were also irradiated in the absence of mRNA for 30 min. (lanes 5,9). The reaction mixtures were electrophoresed on a 7% acrylamide/0.25% agarose gel which contained 7 $\underline{M}$ urea at 500 V for 4 hrs. The gel autoradiographed at -80°C for 16 hrs.

45 The top of the gel and the positions of α and β globin mRNA are indicated along the side of the autoradiogram.

The effects of d-AMTpTpGTTGGTC and d-AMTpTpGCACCAT on cell-free translation of rabbit globin mRNA in a reticulocyte lysate system are shown in Table III. The oligomer and mRNA were irradiated for 60 min. at 0°C prior to translation, which was carried out at 37°C. Little or no inhibition of translation by the

50 oligomers was observed in the absence of irradiation. Translation of globin mRNA irradiated in the presence of as little as 0.5 $\mu$$\underline{M}$ oligomer was inhibited to a significant extent. Inhibition of both α and β globin mRNA translation was observed. This lack of. specificity may reflect the coordination of translation of α and β globin mRNA in the reticulocyte lysate. In any case, the inhibition does not appear to be due to degradation of mRNA. Irradiation of globin mRNA in the presence of 10 $\mu$$\underline{M}$ oligomer for 60 min. does not result in any

55 detectable hydrolylsis of the mRNA as assayed by polyacrylamide gel electrophoresis.

TABLE III.

Effects of AMT-Derivatized Oligonucleoside Methylphosphonates on Translation of Rabbit Globin mRNA in a Rabbit Reticulocyte Lysate at 37°C[a]

Oligomer	Concentration μM	Irradiation 365 nm	%Inhibition[b] α globin	β globin
d-AMTpTpGTTGGTC	2.5	−	12	7
	10.0	−	3	-13
	0.5	+	23	14
	2.5	+	47	43
	10.5	+	30	50
d-AMTpTpGCACCAT	10.0	−	9	13
	0.5	+	18	28
	2.5	+	48	49
	10.0	+	42	34

(a)   The oligomer and globin mRNA (0.04 to 0.08 μM) were irradiated at 0°C for 60 min prior to translation at 37°C.

(b)   A minus sign (-) indicates stimulation of translation.

50   The above results demonstrate that AMT derivatized oligonucleoside methyphosphonates can photocrosslink with mRNA in a sequence specific manner, even at very low oligomer concentrations and thus inhibit mRNA translation. By adjusting the linker length and oligomer chain length it is possible to optimize the specificity of the crosslinking reaction for any particular situation. Furthermore, since the AMT-
55   derivatized oligomers appear to be taken up intact by mammalian cells in culture, the oligomers should be useful to inhibit the translation of virus mRNA in virus-infected cells. Tests with Vesicular Stomatitis virus (VSV) indicate that an AMT-derivatized oligomer complementary to VSV N protein mRNA photocrosslinks with VSV mRNA but not rabbit globin mRNA.

Example 6

Using the method of Chu et al (Proc. Natl. Acad. Sci. USA, 82, 963-967, 1985), the EDTA-derivatized methylphosphonate oligomer shown in Figure 10 was prepared. This oligomer is complementary to
5 nucleotides 60-67 in the coding region of rabbit α globin mRNA. The stability of the oligomer was examined in aqueous solution. The oligomer was found to be stable indefinitely in aqueous ethanol solutions at -20°C. In tris buffer at pH 8.0, the oligomer undergoes autodegradation in the presence of 4 m$\underline{M}$ dithiothreitol and 10 $\mu\underline{M}$ ferrous sulfate. No degradation was observed in the presence of $FeSO_4$ alone or DTT alone. The halflife of autodegradation reaction was approximately 30 min. at room temperature. It appears that
10 autodegradation involves cleavage of the single phosphodiester linkage in the oligomer. Little or no cleavage of the methylphosphonate linkages was observed.

In preliminary experiments relating to the effect of this oligomer on rabbit globin mRNA the oligomer was incubated with the mRNA in the presence or absence of $FeSO_4$/DTT at 0° or 25°C. The mRNA was then electrophoresed on a polyacrylamide gel and the gel then probed with [^{32}p]-labeled oligonucleotides
15 complementary to the initiation codon regions of α or β globin mRNA. Under these conditions there appeared to be no significant hydrolysis (10% or more) of the α globin mRNA.

Example 7

20

Effect of EDTA-Modified d-TpGCACCAT on Rabbit Globin mRNA

This Example describes the preparation of an EDTA-derivatized oligodeoxyribonucleoside methyl-phosphonate starting with an octamer, d-TpGCACCAT. The general structure of the EDTA-modified product
25 is shown in Figure 11.

The octamer d-TpGCACCAT, which is complementary to nucleotides 37-43 of α-globin mRNA and nucleotides 54-61 of β-globin mRNA, was converted to its EDTA derivative by the method of Chu and Orgel (Proc. Natl. Acad. Sci., 82, 9§© (1985)) as shown in Figure 12. The EDTA-pTpGCACCAT. was purified by polyacrylamide gel electrophoresis.
30 The derivatized oligomer was incubated with rabbit globin mRNA and the reaction mixture was subjected to agarose gel electrophoresis. More specifically, reaction mixtures containing 0.25 $\mu\underline{M}$ rabbit globin mRNA were treated as follows: lane 2-no treatment; lane 3-incubated with 1$\underline{M}$ aqueous piperidine at 37°C for 60 min.; lanes 4 and 5-incubated with 0.1 $\mu\underline{M}$ oligomer at 22° for 60 min.; lanes 6 and 7-incubated with 0.1 $\mu\underline{M}$ oligome— at 37°C for 60 min. After incubation, the reaction mixtures were applied to an
35 agarose gel and electrophoresed. The gel was stained with ethidium bromide and photographed. The results are shown in Figure 13.

As shown in Figure 13, under the indicated conditions, the mRNA was hydrolyzed by the oligomer. Significantly more hydrolysis occurred at 22°C (lanes 4 and 5) than at 37°C (lanes 6 and 7). This behavior is consistent with the abilityof the oligomer to bind more strongly to the mRNA at lower temperatures. In
40 these experiments, the mRNA concentration was 0.25 $\mu\underline{M}$ whereas the oligomer concentration was 0.1 $\mu\underline{M}$. The complete hydrolysis of the mRNA observed at 22°C thus shows that oligomer functions in a catalytic manner at very low concentrations. The results of these experiments indicate that EDTA-Fe^{2+} derivatized oligodeoxyribonucleoside methylphosphonates are very effective inhibitors of mRNA function even at low (<1 $\mu\underline{M}$) concentrations. This indicates considerable potential of the derivitized oligomers for use as
45 chemotherapeutic agents.

It will be appreciated from the foregoing that various modifications may be made in the invention as exemplified above. Thus, for example, the chain length of the oligomers may be varied as desired and the nature and positioning of the crosslinking or cleaving group may also be varied. For example, the effectiveness of the derivative may be changed by varying the positioning of the EDTA or psoralen group
50 so that the group is placed either at the 5'-end of a methylphosphonate oligomer which contains no phosphodiester group or by placing the group in the middle or at the 3'-end of the oligomer.

Various available procedures may also be used to prepare the derivatives of the invention. Thus, for example, the synthesis of methylphosphonate oligomers derivatized at their 5'-end with psoralen may be accomplished by first preparing the imidazolid of the oligonucleoside methylphosphonate by reaction with
55 imidazole and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide following the procedure of Chu et al (Nucleic Acid Res., 11, 6513-6529). The imidazolide is then reacted with aminomethyltrimethylpsoralen (AMT) to give the desired product. This series of reactions takes approximately two days to complete. It may be occasionally found that conversion of oligonucleoside methylphosphonate to the desired product is in-

complete. Because the oligomer and product have the same net charge. it is difficult to separate them by gel electrophoresis or DEAE cellulose chromatography. However, it has been found that d-pNp(Np)$_n$N can be converted directly to its aminomethyltrimethylpsoralen derivative, d-AMT-pNp(Np)$_n$ N, by reaction with aminomethyltrimethylpsoralen and carbodiimide in 2,6-lutidine buffer, pH 7.0. The reaction is essentially complete within 6 hours at room temperature. The product is readily purified by DEAE cellulose chromatography.

Using this procedure aminomethyltrimethylpsoralen derivatives, d-AMT-pNp(Np)$_n$N, have been synthesized on a 60 nmol scale with an overall isolated yield of 60% and it is believed that these reactions can be scaled up to at least 1 μmol of starting oligomer to provide approx:mately 600 nmols of derivatized oligomer.

This derivatized oligomer appears to inhibit mRNA translation in the concentration range of 1 to 10 μM. Typically, amounts of the AMT derivatized octanucleoside methylphosphonate, d-AMTpTpGCACCAT, in this range will form a covalent adduct with rabbit globin mRNA when irradiated at 365 nm.

Methods for attaching EDTA or psoralen, or the like to the oligonucleotide are known and these can be used for present purposes to place a variety of functional groups, reactive with RNA, on the selected oligomer sequences. For example, it is known how to attach EDTA covalently to the 5' terminus of a deoxynucleotide sequence and it is also known that the resulting adduct can be used for sequence specific cleavage of DNA. See, for example, papers by Chu et al and Dreyer et al in Proc. Natl. Acad. Sci. USA, Vol. 82, pages 963-967 and 968-972, February 1985.
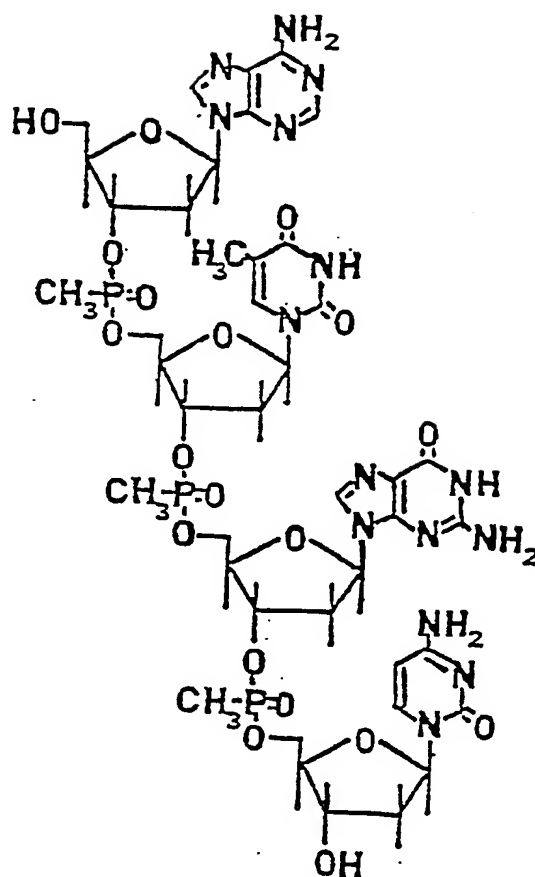
It will be appreciated that the psoralen-and the EDTA-derivatized methylphosphonate oligomers serve to illustrate the broad aspects of the invention. Thus, it is shown that the psoralen derivative, or its equivalent, can be used to covalently crosslink to the target nucleic acid upon activation with ultraviolet light. This can be done to, for example, inhibit translation of mRNA at low concentrations of the oligomer. Likewise, it is shown that the oligomers modified with EDTA or the like can be used to selectively cleave the target nucleic acid and thus render it biologically nonfunctional.

Inasmuch as various modifications may be made in the invention, the scope of the invention is defined in the following claims wherein:

Claims

1. A composition for inactivating a target nucleic acid which comprises an oligonucleoside alkyl or arylphosphonate analogue which is complementary to the sequence of the target nucleic acid and includes a functional group which reacts with the target nucleic acid to render the target nucleic acid inactive, the functional group being one which cleaves the target nucleic acid to inactivate the same.

2. A composition according to claim 1 wherein the phosphonate analogue is an oligonucleoside methylphosphonate.

3. A composition according to claim 1 wherein the functional group is derived from a transition metal chelating complex.

4. A composition according to claim 3 wherein the transition metal chelating complex is ethylenediamine tetraacetate.

5. A composition according to claim 3 wherein the transition metal chelating complex is phenanthroline.

6. A composition according to claim 3 wherein the transition metal chelating complex is a derivative of porphyrin.

7. A composition according to claim 3 wherein the transition metal chelating complex is a derivative of bleomycin.

8. A composition according to claim 1 wherein the functional group is an alkylating agent.

9. A composition according to claim 8 wherein the functional group is an alkyl halide.

10. A composition according to claim 8 wherein the functional group is a haloacetamide.

11. A composition according to claim 8 wherein the functional group is a phosphotriester.

12. A process for inactivating or interferring with the function of undesired or foreign nucleic acid in the presence of otherwise normal living cells which comprises binding the nucleic acid with a derivative of a nonionic oligonucleoside alkyl or arylphosphonate analogue which is complementary to the sequence of the target nucleic acid, said derivatives including a functional group which cleaves the target nucleic acid to prevent the target nucleic acid from functioning.
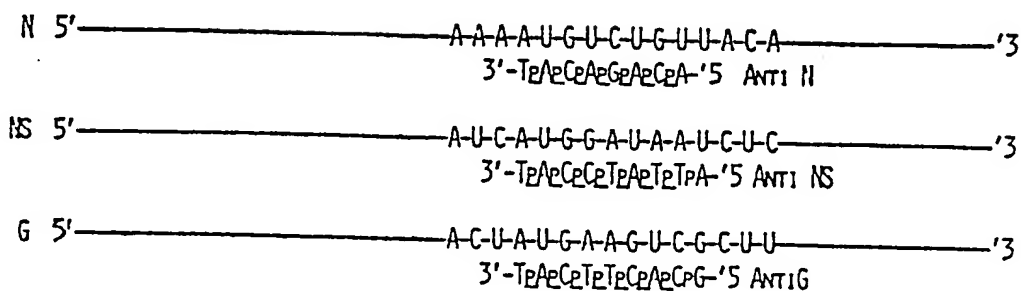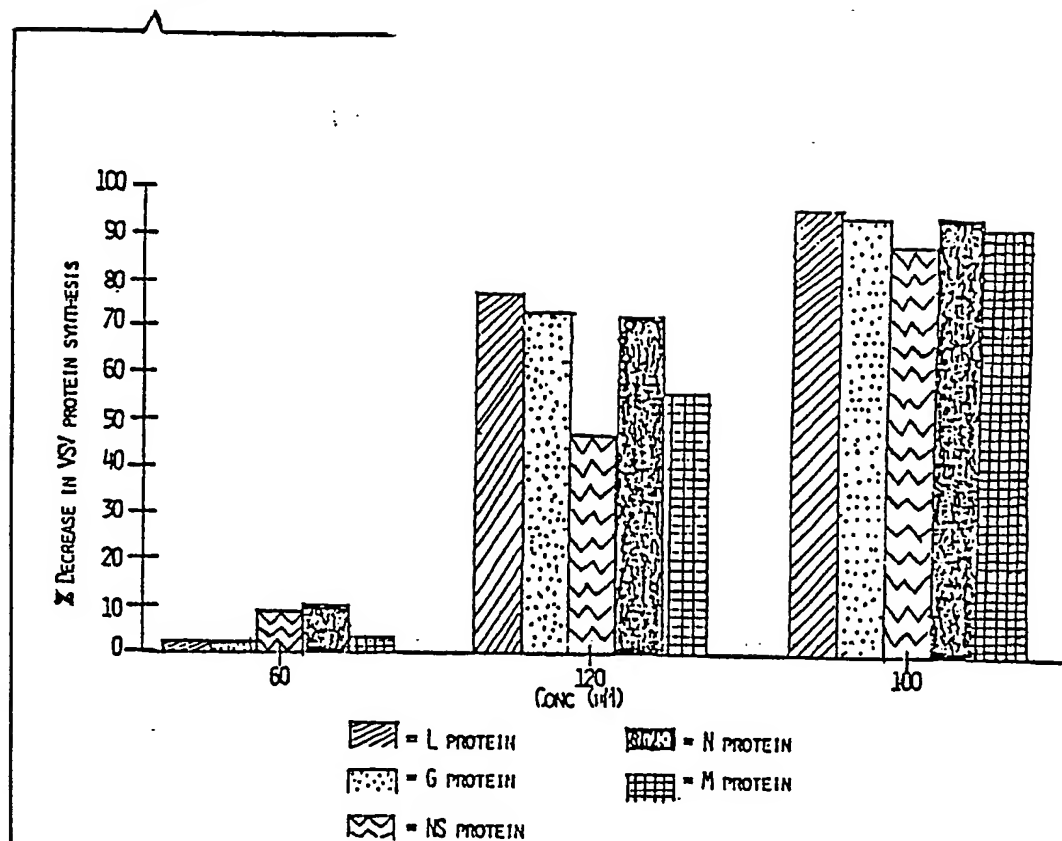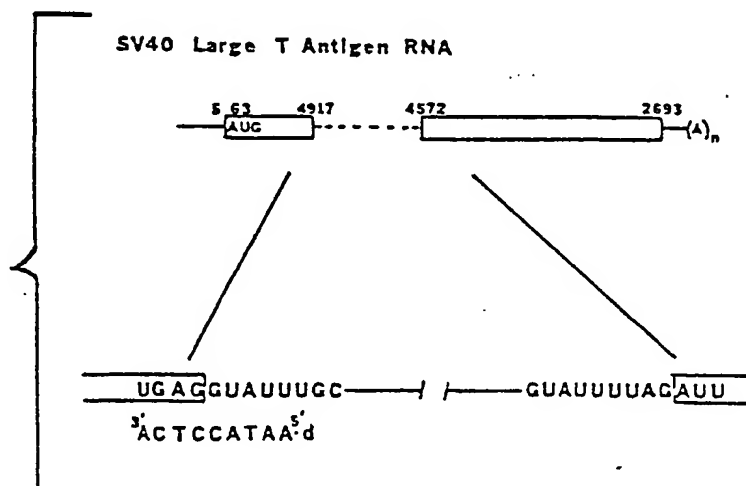
FIG. 1

$d-A_pT_pG_pC$

FIG. 2

N 5'————————————A-A-A-A-U-G-U-C-U-G-U-U-A-C-A————————'3
              3'-TpApCpApGpApCpA-'5 ANTI N

NS 5'————————————A-U-C-A-U-G-G-A-U-A-A-U-C-U-C————————'3
              3'-TpApCpCpTpApTpTpA-'5 ANTI NS

G 5'————————————A-C-U-A-U-G-A-A-G-U-C-G-C-U-U————————'3
              3'-TpApCpTpTpCpApCpG-'5 ANTI G

FIG. 3

Bar chart: Y-axis "% DECREASE IN VSV PROTEIN SYNTHESIS" from 0 to 100. X-axis "CONC (µM)" with values 60, 120, 180 (appears as "100"/180).

Legend:
- ▨ = L PROTEIN
- ⠿ = G PROTEIN
- ⩘ = NS PROTEIN
- ▩ = N PROTEIN
- ▦ = M PROTEIN

FIG. 4

SV40 Large T Antigen RNA

5 63   4917   4572   2693
   AUG   (A)ₙ

UGAGGUAUUUGC———┤ ├——GUAUUUUACAUU

3'ACTCCATAA 5'd

FIG. 5

HN—CH₂

CH₃

CH₃

O

CH₃

NH₂

H₃C

NH

O

CH₃ P O

CH₃ P O

O

NH₂

NH

NH₂

O

NH₂

O

OH

FIG. 6

1 2 3 4 5 6 7

mRNA

FIG. 7a

```
              C  A   A   U
           G        C
      30 U          C
         U            C 40
         C            C
          A        A
           U = A
           U = A
           U = A
           G = C
      G    U = A   G A C A G A      T  A  C
       U            = = = = = =    A  U   G
    20 C           C U G U C U A        G C
       A                        C    U A
       A               A         60 G
              C = G 70          PTpG  G
              A = U                   C
              C = G
      A         |
              U = A
              U = G
              U = G
              U = A
              C = G
         G    U = A
              U = A        C
    m           m m
    G PPP A C A  C    G U
                   80
```

F I G. 7b

dAMTpTpGTTGGTC   dAMTpTpGCACCAT

5'		15'	30'	45'	5'	15'	30'	45'
1		2	3	4	5	6	7	8

—TOP

FIG. 8a

FIG. 8c

5'

T   A
T   A
A   U
G   C
C   G
A   U

3'

T   A

3'

5'

FIG. 8b

d AMTpTpGTTGGTC   dAMTpTpGCACCAT

5'		15'	30'	45'	5'	15'	30'	45'
1		2	3	4	5	6	7	8

┌dAMT(CH₂)₂NHpTpGTTGGTC┐ ┌dAMT(CH₂)₆NHpTpGTTGGTC┐

0'   5'   15'   30'   5'   15'   30'

1   2   3   4   5   6   7   8   9

—TOP

— α
— P

FIG. 9

d-EDTApTpGTTGGTC

FIG. 10

FIG. 11

General structure of an EDTA-Fe^{2+}-derivatized oligonucleoside methylphosphonate.
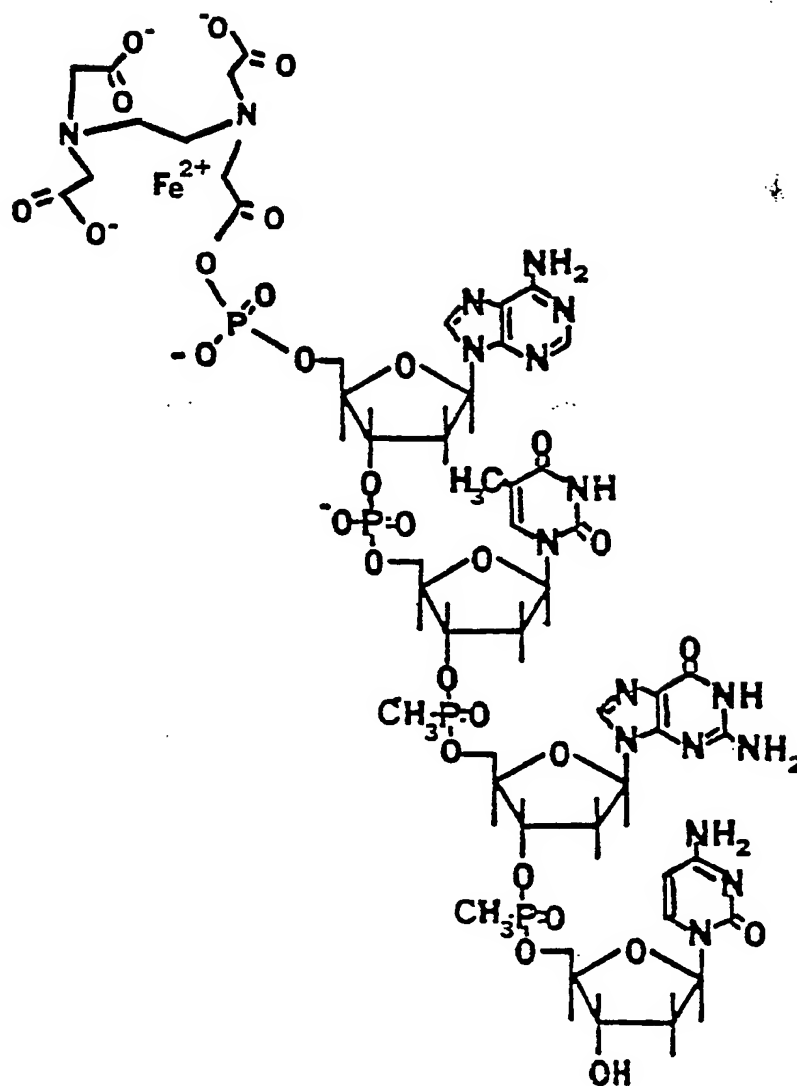
FIG. 12

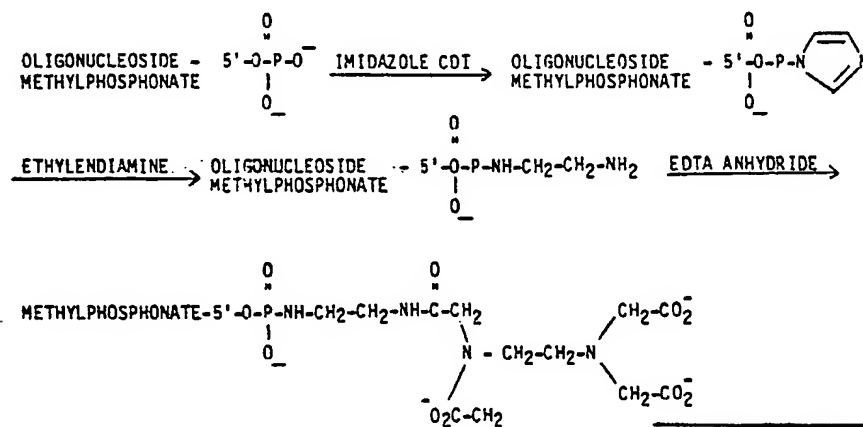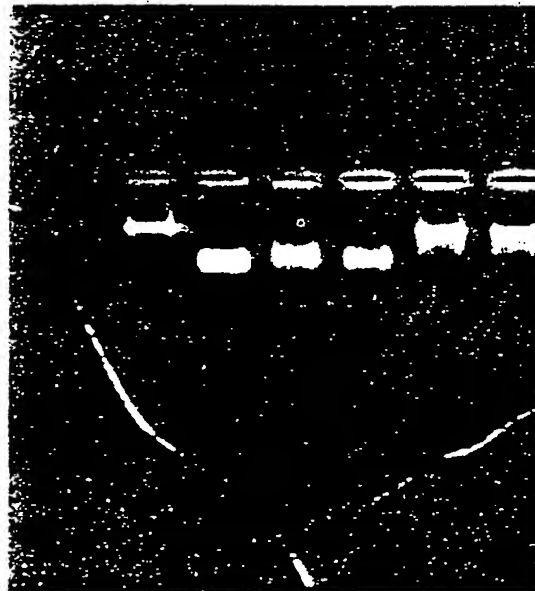Synthetic scheme for the preparation of EDTA-Fe^{2+} modified oligonucleoside methylphosphonates

OLIGONUCLEOSIDE - METHYLPHOSPHONATE   5'-O-P-O$^-$ $\xrightarrow{\text{IMIDAZOLE CDI}}$ OLIGONUCLEOSIDE METHYLPHOSPHONATE - 5'-O-P-N

$\xrightarrow{\text{ETHYLENDIAMINE}}$ OLIGONUCLEOSIDE METHYLPHOSPHONATE - 5'-O-P-NH-CH$_2$-CH$_2$-NH$_2$ $\xrightarrow{\text{EDTA ANHYDRIDE}}$

METHYLPHOSPHONATE-5'-O-P-NH-CH$_2$-CH$_2$-NH-C-CH$_2$

N - CH$_2$-CH$_2$-N

CH$_2$-CO$_2^-$

CH$_2$-CO$_2^-$

O$_2$C-CH$_2$

FIG. 13

Hydrolysis of rabbit globin mRNA by EDTA-Fe^{2+} modified d-TpGCACCAT.

1   2   3   4   5   6   7